Effects of S6-Strain *Mycoplasma gallisepticum* Inoculation at 10, 22, or 45 Weeks of Age on the Digestive and Reproductive Organ Characteristics of Commercial Egg-Laying Hens[1,2]

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ABSTRACT Experimental inoculation of commercial laying hens with the S6-strain of *Mycoplasma gallisepticum* (S6MG) at 20 wk of age, while being maintained under ideal conditions, has previously been shown to affect the lengths and weights of various portions of the reproductive tract. Two trials were conducted in the current study to compare the effects of S6MG inoculation prior to lay at 10 wk of age, during onset of lay at 22 wk of age, and during lay at 45 wk of age on the digestive and reproductive organs of commercial layers similarly housed and maintained under ideal conditions. In each trial, liver weight, liver moisture and lipid concentration, incidence of fatty liver hemorrhagic syndrome, ovary weight, ovarian mature follicle numbers, weights and lengths of the oviduct and oviductal regions, and weights and lengths of the small intestine and small intestinal regions were examined at 60 wk of hen age. At 60 wk, liver lipid concentration was depressed, and isthmus weight, as a percentage of total oviduct weight, was increased in birds that had been inoculated with S6MG at 45 wk. Alterations in liver lipid content and weight of the isthmal portion of the oviduct may occur in response to S6MG inoculation during the later stages of production in layers housed under ideal conditions.

Key words: follicle, intestine, layer, liver, *Mycoplasma gallisepticum*

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INTRODUCTION

The most common symptoms of *Mycoplasma gallisepticum* (**MG**) infection in adult chickens include swollen sinuses and serous exudates in the nostrils (Grimes and Rosenfeld, 1972; Ley and Yoder, 1997). However, MG strains vary in virulence, tissue tropism, and antigenic makeup (Garcia et al., 1994). The severity of MG infection symptoms also depends on such factors as ammonia concentration in the air, stress, and the presence of other pathogens (Sato et al., 1972; Zander, 1984; Mohammed et al., 1987). The S6-strain of MG (**S6MG**) is considered one of the more virulent strains in the field (Levisohn et al., 1986). When S6MG was directly injected into the air sacs of specific-pathogen-free chickens, lesions were present in up to 90% of the experimental population (Lam et al., 1984).

Deterioration in egg quality has been noted due to infection of the genital tract of chickens inoculated via the yolk sac with S6MG. In that study, 25% of the chickens had gross lesions of the oviduct, whereas 81% displayed microscopic lesions found throughout all parts of the oviduct (Pruthi and Kharole, 1981). Nunoya et al. (1997) reported that S6MG infection caused salpingitis and a 3 to 4% decrease in monthly egg production in layers by 36 wk of age, while housed in a commercial facility. More recent studies showed that under controlled conditions, S6MG inoculation at 10 wk of age produced no deleterious effect on the leukocytic characteristics (Peebles et al., 2004) or on egg production and internal egg and eggshell quality (Parker et al., 2002) of commercial layers. Conversely, an inoculation with S6MG at 20 wk did significantly decrease the weights and lengths of various portions of the reproductive tract (Parker et al., 2003). In a companion article in which the same population of birds was used as in the current study, S6MG inoculation at 45 wk, but not at 10 or 22 wk, significantly reduced eggshell quality in birds after 45 wk (Basenko et al., 2005).

Because low-level MG infections can become exacerbated by the onset and increased rate of egg production (Yoder, 1991), it is important to follow birds over an entire laying cycle. The objective of the current study was to compare the effects of S6MG inoculation at 3 different age periods throughout a complete egg-laying cycle on

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various digestive and reproductive organ characteristics in commercial layers. The 3 age periods at which birds were inoculated were prior to lay (10 wk of age), at onset of lay (22 wk of age), and during lay (45 wk of age). In this study, a controlled environment (limited exposure to bacteria and control of temperature, humidity, and lighting) was chosen. A controlled environment and isolation units were used to reduce or remove as many environmental stressors as possible to more accurately assess the true impact of the organism itself. Determinations and comparisons of these effects and their relationships to performance, as reported in a companion article, may provide vital information about the relative impact of these regimens on age-related performance and physiological changes that the layer undergoes in response to an S6MG challenge.

MATERIALS AND METHODS

Pullet Housing and Management

Two individual trials were conducted in this study. In both trials, 1-d-old Single Comb White Leghorn pullets of a single genetic strain (Hy-Line Variety W-36) were obtained from a commercial source that was monitored and certified free of MG and *Mycoplasma synoviae* (**MS;** National Poultry Improvement Plan and Auxiliary Provisions, 1995). Chicks were vaccinated at 10 d of age for infectious bursal disease via the drinking water. At 12 d and again at 4 wk of age, chicks were vaccinated for Newcastle Disease and infectious bronchitis by the same route. At 9 wk of age in trial 1 and at 6 wk of age in trial 2, 10 randomly selected pullets were bled from the left cutanea ulnea wing vein to obtain blood, and their serum was tested for antibodies to MG and MS using the serum plate agglutination (**SPA**) and the hemagglutination-inhibition (**HI**) tests (Yoder, 1975). At those same times, swabs were also collected from the choanal cleft (Branton et al., 1984) and placed into tubes containing Frey's-based (Papageorgiou medium) broth (Frey et al., 1968) supplemented with an additional 0.15 mg of thallium acetate and 10^6 IU of penicillin-G/mL. Tubes were incubated at 37°C for 30 d or until a phenol red indicator reaction occurred in the media indicating growth. Media samples from tubes that showed growth were inoculated onto Frey's-based agar and incubated at 37°C. Colonies with morphology suggestive of *Mycoplasma* species were examined by an agar plate fluorescent antibody (**FA**) method (Baas and Jasper, 1972) that used direct labeling of colonies stained with antiMG polyclonal antibodies produced in rabbits and labeled with fluorescein isothiocyanate (Kleven, 1981).

Until the pullets were 10 wk of age, they were raised on clean dry litter in a 5.5×6.1 m section of a conventional poultry house at the USDA-ARS-Poultry Research Unit, resulting in an initial flock density of 0.034 m^2/bird. A daily artificial lighting schedule followed a 13L:11D cycle. One 75-W incandescent light bulb was used to illuminate each 8.4 m^2 of floor space, providing a calculated intensity at bird level of 35.5 lx. At 10 wk of age, 11 pullets were randomly assigned to each of 16 (total of 176 pullets) negative pressure fiberglass biological isolation units (1.16 m^2). The units were housed in a previously described poultry disease isolation facility at the same USDA research laboratory (Branton and Simmons, 1992). All birds were wing-banded for purposes of identification and individual data collection. The temperature inside each biological unit was maintained at 25°C.

Layer Housing and Management

Hen numbers in each treatment replicate unit were reduced to 10 per unit (total of 160 pullets) at point-of-lay (18 wk of age) so that bird density was 0.116 m^2/bird for the duration of the trial. There were 4 treatment groups with 4 replicate units per treatment. Treatment groups were sham-inoculated controls, and groups that had received S6MG inoculation at 10, 22, or 45 wk of age. The location of treatments within the isolation facility was different between trials to compensate for possible individual isolation unit effects. Beginning at 18 wk of age, the duration of the artificial lighting schedule was increased 15 min/d until a 16.25L:7.75D cycle was achieved in trial 1, and a 17.25L:6.75D cycle was achieved in trial 2. These respective artificial lighting programs were maintained through the end of both trials.

Pullet and Layer Diets

For the entirety of each trial, chickens had ad libitum access to feed and water. Diets in both trials were formulated according to the age of the birds and included the following: starter (0 to 6 wk), grower (7 to 12 wk), developer (13 to 18 wk), prelay (18 to 19 wk), and layer (20 to 60 wk). All diets were formulated to meet or exceed National Research Council (1994) specifications. Ingredient percentages and calculated analyses of these diets were as described by Burnham et al. (2002a). In both trials, CP and lysine percentages in the layer diet were adjusted according to the percentage of feed consumed per bird every 28 d until trial termination. No medication was administered during the study. At 54 wk of age in trial 2, feed samples were analyzed for determined moisture, ash, CP, crude fat, crude fiber, total nitrogen, total digestible nitrogen, and fatty acid concentrations. Feed sample determined ingredient concentrations were as previously described by Basenko et al. (2005). Feed samples were obtained by mixing 5 samples from 3 depths (2 on the top, 1 in the middle, and 2 at the bottom) within a common feed container. The mixed samples from each of 2 containers, used for feeding all of the birds in the study, were pooled and thoroughly mixed prior to analysis. The pooled samples were stored at –20°C for later analysis. All determined analyses were performed according to the methods of the Association of Official Analytical Chemists (1980).

S6MG Inoculation and Mycoplasma Detection

The S6MG-treated pullets were inoculated via eye drop in the right eye with 0.04 mL of a 24-h Frey's broth culture of high-passage S6MG at 10, 22, or 45 wk of age. *Mycoplasma gallisepticum* organisms were passed in broth medium after being received from S. H. Kleven (University of Georgia, Athens, GA) at the 212th passage. This S6MG strain was tested by a tracheal ring organ culture model as described by Cherry and Taylor-Robinson (1970) and was shown to significantly reduce ciliary activity within 5 d. Titers and passages of the experimental inocula in each trial were as previously described by Basenko et al. (2005). Similarly, pullets designated as controls were sham-inoculated via eye drop in the right eye at 10 wk of age with 0.04 mL of sterile Frey's broth medium. To ensure that all treatment groups were equally stressed by the inoculation process and to minimize the possibility of treatment group cross-contamination, control birds were sham inoculated only once. At 60 wk of age in both trials, one randomly selected hen from each replicate unit in each of the 4 treatment groups was bled and swabbed. Each of these samples was tested for the presence of *Mycoplasma* species as previously described for pullets at 9 wk of age in trial 1 and at 6 wk of age in trial 2.

Data Collection

Both trials were terminated at 60 wk of bird age. At that time in both trials, 2 tagged hens from each replicate unit were weighed, euthanized by cervical dislocation, and their organs removed. In both trials, organ analyses included: liver weight; liver moisture and lipid concentration; incidence of fatty liver hemorrhagic syndrome (**FLHS**); ovary weight, histology, and mature follicle quantification; total oviduct weight and length; weights, lengths, and histologies of the infundibulum, magnum, isthmus, uterus, and vagina; small intestine weight and length; and weights and lengths of the duodenum, jejunum, and ileum. Liver, ovary, oviduct, and small intestine weights were expressed as percentages of BW. The FLHS incidence was expressed as the percentage of birds exhibiting FLHS to any degree. Weights of the various regions of the oviduct and small intestine were expressed as percentages of both BW and total organ weight. Lengths of the various regions of the oviduct and small intestine were expressed as percentages of total organ length.

Mature Follicle Quantification

Mature follicle quantification was as described by Burnham et al. (2002b). Briefly, in both trials, the entire ovary was removed and the number of mature (diameter ≥12 mm) yellow ovarian follicles was recorded for each bird. A caliper was used to measure follicle diameter. Based on the number of mature ovarian follicles present in each bird, a categorical number from zero to 6 was assigned. The mean number of mature ovarian follicles and the percentage of birds having 0, 1, 2, 3, 4, 5, or 6 mature follicles in each replicate unit were calculated.

Liver Moisture and Lipid Analysis

For analysis of liver moisture content, fresh liver samples (2 g) were dried according to the procedure of Peebles et al. (1999) in a commercial oven (Model EL20, General Electric Co., Chicago Heights, IL). Liver moisture content was calculated as the difference between the fresh and dry weights of the sample and was expressed as a percentage of fresh liver sample weight. For analysis of liver lipid content, lipid was extracted from fresh liver samples (3 g) according to the procedure previously described by Bligh and Dryer (1959) and as modified by Latour et al. (1998). Liver lipid content was expressed as a percentage of total fresh liver sample weight.

Histopathologic Examination

Upon termination of each trial, one tissue sample from the ovary, infundibulum, magnum, isthmus, uterus, and vagina was harvested from 1 hen in each of 4 units in both control and treatment groups. Tissue samples were placed in 10% buffered neutral formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Each tissue sample was observed and scored for the presence or absence of lymphoid and heterophil infiltrates as described by Branton et al. (2000). Treatment assignments were unknown to the evaluator.

Statistical Analysis

Individual sample data within each replicate unit were averaged, and angular transformations (arc sine of the square root of the proportion affected) were performed on all percentage data prior to analysis. A completely randomized experimental design was used, and data were analyzed by 1-way ANOVA using the MIXED Procedure of SAS, version 8.1 (2000). Trial, replication within trial, and treatment × replication within trial were taken as random effects. Least squares means were compared in the event of significant global effects (Steel and Torrie, 1980). Statements of significance were based on $P \leq 0.05$, unless otherwise stated.

RESULTS

All initial mycoplasmal cultures as well as SPA and HI test results obtained from pullets at 9 wk of age in trial 1 and at 6 wk of age in trial 2 were negative for MG and MS. Serum samples obtained from control birds at 60 wk of age in both trials were SPA and HI negative for MG, and the same tests were positive for MG in the S6MG-inoculated hens. Hens were considered MG-free when they exhibited no detectable HI titers. All S6MG-inoculated hens tested had HI titers ≥1:80 (geometric mean of 80.0). Similarly, FA culture results for swabs obtained at 60 wk of age in both trials were negative for *Mycoplasma*

Table 1. Body weight, liver lipid concentration, and relative isthmus weight (percentage of total oviduct weight) at 60 wk of age in sham-inoculated control and in 10 (S6MG-10), 22 (S6MG-22), and 45 (S6MG-45) wk S6MG inoculation treatments, trials 1 and 2[1]

Treatment	Body weight (g)	Liver lipid (%)	Isthmus weight (%)
Control	1,517	10.1[a]	10.2[b]
S6MG-10	1,436	9.6[a]	10.1[b]
S6MG-22	1,477	10.4[a]	10.5[ab]
S6MG-45	1,397	7.2[b]	11.1[a]
SEM[2]	45	1.2	0.5

[a,b]Means among treatments within column with no common superscript differ significantly ($P \leq 0.05$).

[1]n = 4 replicate units for the mean of each parameter within each treatment. S6MG = S6-strain of *Mycoplasma gallisepticum*.

[2]SEM based on pooled estimate of variance.

species growth for all control hens tested, whereas all S6MG-inoculated hens tested were MG positive (positive for MG fluorescence) and MS negative (negative for MS fluorescence).

At wk 60, there were no significant differences among treatments for BW, relative liver weight, liver moisture content, or incidence of FLHS. However, liver lipid concentration was very highly significantly ($P \leq 0.0001$) affected by treatment (Table 1). Liver lipid content was significantly lower at 60 wk in birds that had been inoculated at 45 wk with S6MG when compared with controls and birds that had been inoculated with S6MG at either 10 or 22 wk. There were no significant differences in liver lipid content among the sham control-inoculated, 10 wk S6MG-inoculated, and 22 wk S6MG-inoculated groups. Although BW at wk 60 was not significantly affected by treatment, these data are included in Table 1 for reference.

Weights of the ovary, oviduct, and regions of the oviduct, including the infundibulum, magnum, isthmus, uterus, and vagina, when expressed as percentages of BW at 60 wk, were not significantly affected by treatment. Furthermore, weights and lengths of the infundibulum, magnum, uterus, and vagina, as percentages of total oviduct weight and length, respectively, were not significantly affected by treatment. However, isthmus weight, as a percentage of total oviduct weight, was significantly ($P \leq 0.05$) affected by treatment (Table 1). Isthmus weight relative to that of the total oviduct was greater at 60 wk of age in birds that had been inoculated with S6MG at 45 wk compared with controls and those inoculated with S6MG at 10 wk. The relative isthmus weights of birds inoculated at 22 wk were not significantly different from any of the other treatment groups.

Mean mature ovarian follicle number and the percentages of birds with 0, 1, 2, 3, 4, 5, or 6 mature follicles at 60 wk was not significantly different between treatment groups. Also, weights of the small intestine and its regions, including the duodenum, jejunum, and ileum, when expressed as percentages of BW, were not significantly affected by treatment. Weights and lengths of regions of the small intestine, including the duodenum, jejunum, and ileum, as percentages of total small intestine weight and length, respectively, were likewise not significantly affected by treatment.

DISCUSSION

At the end (60 wk of age in both trials) of this study, SPA, HI, and FA tests verified systemic infections in S6MG-inoculated birds. Conversely, sham-inoculated birds remained S6MG-free throughout the study. Manifestations of MG usually occur in the respiratory system, and lesions become extensive when complicated by other bacteria. Furthermore, environmental factors such as dust and ammonia, along with intensive rearing or stress, crowding, cold weather, live virus vaccination, or natural virus infection may also be important in lesion incidence and severity (Jordan, 1972; Springer et al., 1974; Jordan, 1985). However, when there are no secondary infections in association with mycoplasmosis, infection is often subclinical or mild (Kerr and Olson, 1967). The birds in this study were housed in biological isolation units, from 10 wk of age through the remainder of the study, where they remained discernibly free of natural infections and other environmental stressors common in commercial operations. As a result, the S6MG-infected hens exhibited no outward pathological symptoms. Furthermore, as reported by Basenko et al. (2005), S6MG inoculation treatment had no significant effect on either the mortality or BW of these same hens.

Burnham et al. (2002a) showed that egg production was both delayed and reduced and that egg characteristics were altered in layers inoculated with the F-strain of MG at 12 wk. It was later suggested by Burnham et al. (2002b) that alterations in the performance and egg characteristics of those layers were related to mutual functional disturbances in the liver, ovary, and oviduct, without concomitant intestinal changes. However, Parker et al. (2002) showed that 10-wk S6MG inoculations of commercial layers housed similarly to those by Burnham et al. (2002a,b) had no effect on BW, egg production, egg weight, associated internal egg and eggshell quality parameters, or histopathologic lesion scores. In a subsequent report (Peebles et al., 2004), the leukocyte profile of the same birds used by Parker et al. (2002) was further shown not to be affected by the 10-wk S6MG inoculation. Parker et al. (2003) later noted that the inoculation of commercial layer hens at 20 wk of age with S6MG had no effect on BW, egg production, digestive tract weight and length, or histopathologic lesion score. Nevertheless, similar to the findings of Burnham et al. (2002b), significant differences were noted in the lengths and weights of various portions of the reproductive tract due to the inoculation of S6MG at 20 wk of age (Parker et al., 2003). These results demonstrated that S6MG can alter the reproductive tract without subsequently affecting overall performance. Similar to the results of Parker et al. (2003), the results of the current study included an effect of S6MG on the characteristics of the oviduct. However, only the 45-wk S6MG inoculation affected the reproductive tract in these birds by increasing isthmus weight, as a percentage of total

oviduct weight, in comparison to controls. A significant increase in the relative weight of the isthmus may be due to small changes in various regions of the oviduct, which may have included an increase in isthmal weight in association with small decreases in the weight of one or more of the other regions.

In the current study, the lack of any effects on the oviduct from either the 10- or 22-wk S6MG inoculations in contrast to those of the 45-wk inoculation indicates that differences in the timing of inoculation (10 and 22 vs. 45 wk) may lead to differential effects on the oviduct and its various regions. Analogous to Parker et al. (2002, 2003), Basenko et al. (2005) showed that S6MG inoculation at 10 and 22 wk of age had no effect on BW, egg production, egg weight, or on internal egg and eggshell quality parameters. But in contrast, a 45-wk inoculation did alter eggshell quality despite the absence of any subsequent effects on egg production (Basenko et al., 2005). As was found after the 20-wk S6MG inoculation administered by Parker et al. (2003), the 45-wk inoculation likewise altered the oviduct without affecting egg production. Nevertheless, upon having examined the effects of S6MG inoculation timing on eggshell quality, Basenko et al. (2005) suggested that the birds may be better able to adapt to an S6MG infection when inoculated prior to or at the onset of lay rather than late in lay. The suggestion by Basenko et al. (2005) might also partially explain the disparity in effects between the 45-wk inoculation and those at 10 or 22 wk on the reproductive tract characteristics of the layers in the current study. The possibility also exists that the birds inoculated at 10 or 22 wk may have likewise experienced oviductal changes, but that these changes were unapparent by wk 60 due to the much longer interval of time between inoculation and necropsy in comparison with those inoculated at 45 wk. Flock differences may account for the contrast in the range of effects on the oviduct as a result of the 22-wk inoculation in this study and the 20-wk inoculation of Parker et al. (2003).

A possible influence of the longer gap in time between inoculation and necropsy for birds inoculated at 10 or 22 wk compared with those inoculated at 45 wk exists for liver lipid content as well as for characteristics of the reproductive tract. Nevertheless, a decrease in liver lipid content in response to the 45-wk inoculation compared with the absence of an effect after the 10- or 22-wk inoculations in this study further suggests that livers are more susceptible to the effects of S6MG infection at 45 wk than at prelay or at the initiation of lay in commercial egg-laying hens. Recent findings showing that MG has the ability to invade cells (Winner et al., 2000) suggests that MG may be capable of interfering with liver lipid metabolism. Furthermore, Winner et al. (2000) demonstrated that MG has the capability of passing through the mucosal barrier to cause systemic infections, and *Mycoplasma* species may be cultured from the avian liver (Sahu and Olson, 1976). Therefore, the active infection may cause the livers of birds infected with MG to react earlier in the reproductive cycle in ways similar to that of inefficient or aging hens.

In conjunction with the relative increase in isthmus weight and decrease in liver lipid content in the current study, Basenko et al. (2005) reported that these same birds also experienced reductions in eggshell weight per unit surface area and percentage eggshell weight across wk 47 and 58 in response to the 45-wk S6MG inoculation. These associated responses would imply that alterations in liver metabolism and the characteristics of various regions of the oviduct after an S6MG inoculation at 45 wk might influence subsequent eggshell quality. The lack of any significant changes in the relative lengths of the various regions of the oviduct due to treatment would suggest that the duration of time allocated to each region for the egg formation process was not altered. Nevertheless, should an increase in relative isthmus weight after the 45-wk S6MG inoculation have been due primarily to an increase in the absolute weight of the isthmus, it may have had a negative impact on eggshell quality. Because the formation of mammillary cores, which are the epitactic centers for eggshell calcification, are initiated in the distal portion of the isthmus (Johnson, 2000), a disproportionate thickening of the epithelium in the isthmus may have obstructed mammillary core development, which in turn may have disrupted subsequent eggshell deposition. The absence of associated changes in eggshell quality and reproductive tract characteristics after inoculations prelay or at onset of lay in comparison with concomitant changes in both eggshell quality and reproductive tract characteristics after a 45-wk (during lay) inoculation would also suggest that the impact of S6MG inoculations on the eggshell via the oviduct are greatest when given during lay.

In conclusion, only the 45-wk S6MG inoculation altered the characteristics of the reproductive tracts of the birds in this study. The 45-wk S6MG inoculation also caused concomitant reductions in eggshell quality, as reported in the companion study by Basenko et al. (2005). Perhaps possible changes in the reproductive tracts of birds inoculated at 10 or 22 wk became no longer apparent by 60 wk, or the birds were better able to adapt to an S6MG infection when inoculated prior to or at the onset of lay rather than late in lay. In addition, reproductive tract alterations in response to S6MG may become manifested through changes in eggshell quality only in older birds that are experiencing declines in production when housed under ideal conditions. However, because these birds were housed in biological isolation units, these results do not preclude the possibility that additional performance parameters may be affected throughout an entire laying cycle in birds infected with S6MG before and during lay that are housed in facilities where there are increased levels of environmental stress.

REFERENCES

Association of Official Analytical Chemists. 1980. Official Methods of Analysis. 14th ed. Assoc. Off. Anal. Chem., Washington, DC.

Baas, E. J., and D. E. Jasper. 1972. Agar block technique for identification of Mycoplasmas by use of fluorescent antibody. Appl. Microbiol. 23:1097–1100.

Basenko, E. Y., E. D. Peebles, S. L. Branton, S. K. Whitmarsh, and P. D. Gerard. 2005. Effects of S6-strain *Mycoplasma gallisepticum* inoculation at 10, 22, or 45 weeks of age on the performance characteristics of commercial egg laying hens. Poult. Sci. 84:1663–1670.

Bligh, E. G., and W. J. Dryer. 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37:911–913.

Branton, S. L., H. Gerlach, and S. H. Kleven. 1984. *Mycoplasma gallisepticum* isolation in layers. Poult. Sci. 63:1917–1919.

Branton, S. L., B. D. Lott, J. D. May, W. R. Maslin, G. T. Pharr, S. D. Bearson, S. D. Collier, and D. L. Boykin. 2000. The effects of ts-11 strain *Mycoplasma gallisepticum* vaccination in commercial layers on egg production and selected egg quality parameters. Avian Dis. 44:618–623.

Branton, S. L., and J. D. Simmons. 1992. Design of a poultry disease isolation facility with programmable environmental control. Appl. Eng. Agric. 8:695–699.

Burnham, M. R., S. L. Branton, E. D. Peebles, B. D. Lott, and P. D. Gerard. 2002a. Effects of F-strain *Mycoplasma gallisepticum* inoculation at twelve weeks of age on performance and egg characteristics of commercial egg laying hens. Poult. Sci. 81:1478–1485.

Burnham, M. R., E. D. Peebles, S. L. Branton, M. S. Jones, P. D. Gerard, and W. R. Maslin. 2002b. Effects of F-strain *Mycoplasma gallisepticum* inoculation at twelve weeks of age on digestive and reproductive organ characteristics of commercial egg laying hens. Poult. Sci. 81:1884–1891.

Cherry, J. D., and D. Taylor-Robinson. 1970. Growth and pathogenesis of *Mycoplasma mycoides* var. capri in chicken embryo tracheal organ cultures. Infect. Immun. 2:431–438.

Frey, M. C., R. P. Hanson, and D. P. Anderson. 1968. A medium for the isolation of avian Mycoplasma. Am. J. Vet. Res. 29:2164–2171.

Garcia, M., M. G. Elfaki, and S. H. Kleven. 1994. Analysis of the variability in expression of *Mycoplasma gallisepticum* surface antigens. Vet. Microbiol. 42:147–158.

Grimes, T. M., and L. E. Rosenfeld. 1972. Experimental respiratory disease and airsacculitis in fowls caused by MG. Aust. Vet. J. 48:113–116.

Johnson, A. L. 2000. Reproduction in the female. Pages 569–596 in Sturkie's Avian Physiology, 5th ed. G. C. Whittow, ed. Acad. Press, San Diego, CA.

Jordan, F. T. 1972. The epidemiology of disease of multiple aetiology: The avian respiratory disease complex. Vet. Rec. 90:556–562.

Jordan, F. T. 1985. Gordon memorial lecture: People, poultry and pathogenic mycoplasmas. Br. Poult. Sci. 26:1–15.

Kerr, K. M., and N. O. Olson. 1967. Pathology in chickens experimentally inoculated or contact-infected with *Mycoplasma gallisepticum.* Avian Dis. 11:559–578.

Kleven, S. H. 1981. Transmissibility of the F-strain of *Mycoplasma gallisepticum* in leghorn chickens. Poult. Sci. 25:1005–1018.

Lam, K. M., J. Rosen, and H. E. Adler. 1984. Temperature-sensitive mutants of *Mycoplasma gallisepticum.* J. Comp. Pathol. 94:1–8.

Latour, M. A., E. D. Peebles, S. M. Doyle, T. W. Smith, and C. R. Boyle. 1998. Broiler breeder age and dietary fat influence the yolk fatty acid profiles of fresh eggs and newly hatched chicks. Poult. Sci. 77:47–53.

Levisohn, S., M. J. Dykstra, M. Y. Lin, and S. H. Kleven. 1986. Comparison of in vivo and in vitro methods for pathogenicity evaluation for *Mycoplasma gallisepticum* in respiratory infection. Avian Pathol. 15:233–246.

Ley, D. H., and H. W. Yoder. 1997. *Mycoplasma gallisepticum* infection. Pages 194–207 in Diseases of Poultry, 10th ed., B. W. Calnek, ed. Iowa State Univ. Press, Ames, IA.

Mohammed, H. O., T. E. Carpenter, and R. Yamamoto. 1987. Evaluation of factors associated with infection of commercial layers with *Mycoplasma gallisepticum* and *M. synoviae.* Avian Dis. 31:470–476.

National Poultry Improvement Plan and Auxiliary Provisions. 1995. Published in Chapter 9 Code of Federal Regulations (9CFR). USDA-APHIS-VS, Conyers, GA.

National Research Council. 1994. Nutrient Requirements of Poultry. 9th rev. ed. Natl. Acad. Press, Washington, DC.

Nunoya, T., K. Kanai, T. Yagihashi, S. Hoshi, K. Shibuya, and M. Tajima. 1997. Natural case of salpingitis apparently caused by *Mycoplasma gallisepticum* in chickens. Avian Pathol. 26:391–398.

Parker, T. A., S. L. Branton, M. S. Jones, E. D. Peebles, P. D. Gerard, K. O. Willeford, M. R. Burnham, and W. R. Maslin. 2002. Effects of an S6 strain of *Mycoplasma gallisepticum* challenge before beginning of lay on various egg characteristics in commercial layers. Avian Dis. 46:593–597.

Parker, T. A., S. L. Branton, M. S. Jones, E. D. Peebles, P. D. Gerard, K. O. Willeford, G. T. Pharr, and W. R. Maslin. 2003. Effects of an S6 strain of *Mycoplasma gallisepticum* challenge at onset of lay on digestive and reproductive tract characteristics in commercial layers. Avian Dis. 47:96–100.

Peebles, E. D., L. Li, S. Miller, T. Pansky, S. Whitmarsh, M. A. Latour, and P. D. Gerard. 1999. Embryo and yolk compositional relationships in broiler hatching eggs during incubation. Poult. Sci. 78:1435–1442.

Peebles, E. D., T. A. Parker, S. L. Branton, K. O. Willeford, M. S. Jones, P. D. Gerard, G. T. Pharr, and W. R. Maslin. 2004. Effects of an S6 strain of *Mycoplasma gallisepticum* inoculation before beginning of lay on the leukocytic characteristics of commercial layers. Avian Dis. 48:196–201.

Pruthi, A. K., and M. U. Kharole. 1981. Sequential pathology of genital tract in chickens experimentally infected with *Mycoplasma gallisepticum.* Avian Dis. 25:768–778.

Sahu, S. P., and N. O. Olson. 1976. Use of the agar-gel precipitin test to evaluate broiler breeder and commercial layer flocks for *Mycoplasma gallisepticum* infection. Avian Dis. 20:563–573.

SAS Inst., Inc. 2000. SAS Proprietary Software Release 8.1. SAS Institute, Inc., Cary, NC.

Sato, S., S. Shoya, and H. Kobayashi. 1972. Effect of ammonia on *Mycoplasma gallisepticum* infection in chickens. Natl. Inst. Anim. Health Quart. 13:45–53.

Springer, W. T., C. Luskus, and S. S. Pourciau. 1974. Infectious bronchitis and mixed infections of *Mycoplasma synoviae* and *Escherichia coli* in gnotobiotic chickens. Synergistic role in the airsacculitis syndrome. Infect. Immun. 10:578–589.

Steel, R. G. D., and J. H. Torrie. 1980. Principles and Procedures of Statistics. A Biometrical Approach. 2nd ed. McGraw-Hill, New York, NY.

Winner, F., R. Rosengarten, and C. Citti. 2000. In vitro cell invasion of *Mycoplasma gallisepticum.* Infect. Immun. 68:4238–4244.

Yoder, H. W. Jr., 1975. Mycoplasmosis: Isolation and Identification of Avian Pathogens. Am. Assoc. Avian Path., College Station, TX.

Yoder, H. W. Jr., 1991. Mycoplasmosis. Pages 196–231 in Diseases of Poultry, 8th ed. B. W. Calnek, H. J. Barnes, C. W. Beard, W. M. Reid, and H. W. Yoder, ed. Iowa State Univ. Press, Ames, IA.

Zander, D. V. 1984. Principles of disease prevention. Pages 1–37 in Diseases of Poultry, 8th ed., M. S. Hofstad, H. J. Barnes, B. W. Calnek, W. M. Reid, and H. W. Yoder Jr., ed. Iowa State Univ. Press, Ames, IA.